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Interaction of Tacrine at M_1 and M_2 Cholinoceptors in Guinea Pig Brain

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Abstract
Tacrine (THA) selectively modulates binding of M_1 ligands in an allosteric fashion causing positive cooperativity. The binding affinity of THA to M_1 and M_2 cholinoceptors is similar. It is therefore proposed that the allosteric selectivity of THA is a function of the binding site and not of THA itself. Its interaction of M_1 and M_2 cholinoceptors was examined in guinea pig brain homogenates using the selective M_1 and M_2 antagonists [^{3}H]-pirenzepine ([^{3}H]PZ) and [^{3}H]AF-DX 384. The dissociation constants were 0.36 nmol/l for the M_1 receptor and 0.23 nmol/l for the M_2 receptor. We also compared the binding of THA and methoctramine (MTA) at M_2 receptors. Tacrine displayed similar binding affinity for both M_1 and M_2 receptor subtypes. MTA was 100 times more potent an inhibitor of [^{3}H]AF-DX 384 binding at M_2 receptors than THA. In addition, THA was found to slow the dissociation of [^{3}H]PZ from the M_1 receptor. In contrast, the dissociation of [^{3}H]AF-DX 384 from M_2 receptor subtypes was unaffected. We conclude that THA acts as an agonist at M_1 cholinoceptors because it slowed the dissociation of [^{3}H]PZ. At M_2 cholinoceptors its nature is that of an antagonist because it had no effect on [^{3}H]AF-DX 384 dissociation.

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Introduction

Tacrine (1,2,3,4-tetrahydro-9-aminoacridine; THA) has undergone clinical trials for palliative treatment of Alzheimer's disease [1]. While oral doses alleviated some symptoms of the disease, the improvement was temporary and Summers et al. [1] anticipated that THA would cease to have effects as Alzheimer's disease progressed. Therefore, THA's efficacy is yet to be established. Characterising THA's action at M cholinoceptors may add to the understanding of this drug's activity. While the nature of inhibition of cholinesterase by THA is debatable [2], its capacity to mediate multiple effects at the receptor level could have important implications in the treatment of Alzheimer's disease [3]. Several researchers have examined its effects of M_1 and M_2 cholinoceptors with radioligand binding techniques using the selective M_1 antagonist [^{3}H]-pirenzepine ([^{3}H]PZ) [4, 5] and the moderately selective M_2 antagonist [^{3}H]-N-methylscopolamine [6, 7]. In this study, we investigated the effects of THA on the M_2 subtype using the highly selective antagonist [^{3}H]AF-DX 384 [8], a benzodiazepine-substituted compound. The binding characteristics were compared with those for methoctramine (MTA) which is also a potent, selective M_2 antagonist [9]. Finally, the effects of THA at M_1 cholinoceptors were re-examined by competition experiments with [^{3}H]PZ.

Methods

Female guinea pigs (300–400 g) were killed by cervical dislocation and the whole brain removed and stored in liquid nitrogen (−80 °C) until use. For the assay, the brain (2–4 g) was thawed and weighed. It was homogenised in 20 ml ice-cold Tris-HCl (50 mmol/l, pH 7.7) using an Ultra-Turrax homogeniser on setting 6 for two 15-second bursts with a 30-second rest period in between. The homogenate was then centrifuged at 18,800 *g* for 30 min at 4 °C in a Sorvall SS-4 manual centrifuge. The pellet was resuspended in 20 ml of buffer and recentrifuged under the previous conditions. The final pellet was resuspended in 10 ml of buffer for the [^{3}H]PZ assay and 20 ml for the [^{3}H]AF-DX 384 assays. The same procedure was used for the preparation of cerebral cortex and cerebellum for M_1 and M_2 binding studies except that the final pellet was resuspended in a final volume of 10 ml for both radioligands.

The protein concentration of all membrane extracts was 4–10 mg/ml as determined by the Hartree modification of the Lowry protein assay [10].

[^{3}H]PZ (87 Ci/mmol) and [^{3}H]AF-DX 384 (97 Ci/mmol) were both obtained from DuPont (Australia). MTA and (±) quinuclidinylbenzilate (QNB) were obtained from Research Biochemicals, USA. All other materials were of analytical grade and readily available commercially.

Interaction at M cholinoceptors was assessed using a radioligand binding technique. The equilibrium dissociation constant (K_D) and apparent maximum number of binding sites (B_{max}) were determined by incubating varying concentrations of the radioligands ([^{3}H]PZ, 0.004–1.04 nmol/l; [^{3}H]AF-DX 384, 0.005–0.610 nmol/l) at 25 °C with 200 ml homogenate made up to 2 ml with buffer. The incubation period was 2 h for [^{3}H]PZ and 1 h for [^{3}H]AF-DX 384. Non-specific binding was defined using 1 mmol/l atropine sulfate for [^{3}H]AF-DX 384 and 1 mmol/l (±) QNB for [^{3}H]PZ. Binding was terminated by rapid filtration through polyethyleneimine-treated (0.05% w/v) Whatman GF/B filter paper using a Brandel Cell Harvester. The filters were rapidly washed with 15 ml of cold buffer in five aliquots and equilibrated with 10 ml of Beckman Ready Protein scintillator. Radioactivity was measured after 24 h with a Beckman Liquid Scintillation System LS 5801, counting efficiency for tritium being 45%.

For dissociation studies, the membrane was equilibrated with radioligand (0.1 nmol/l [^{3}H]PZ and 0.06 nmol/l [^{3}H]AF-DX 384) by incubating at 25 °C for 60 min prior to the initiation of dissociation by the addition of 1 mmol/l QNB for [^{3}H]PZ and 1 mmol/l atropine sulfate for [^{3}H]AF-DX 384. Fifty to five hundred micromolar THA was used in the [^{3}H]PZ studies and 1–1,000 mmol/l THA was used for [^{3}H]AF-DX 384. The higher concentration (1,000 mmol/l) used for the latter case was to ensure that any allosteric modulation at M_2 cholinoceptor site by THA would not be overlooked. Dissociation was allowed to proceed for a maximum of 90 min, being stopped by filtration at several time intervals (10, 15, 30, 45, 60 and 90 min) and counting as already described.

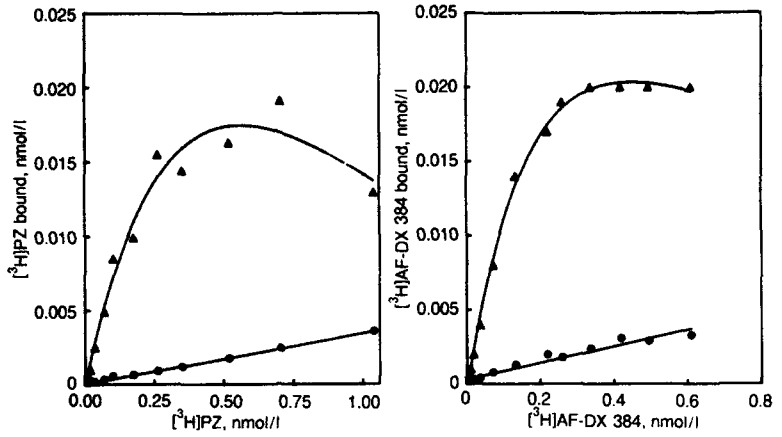

Fig. 1. Representative saturation isotherms of [^{3}H]PZ and [^{3}H]AF-DX 384 binding to guinea pig brain membranes. ● = Non-specific binding; ▲ = specific binding.

Results

Figure 1 depicts the corresponding saturation isotherms for [^{3}H]PZ and [^{3}H]AF-DX 384. The binding of both radioligands to the membranes was saturable. The specific binding of [^{3}H]PZ increased with increasing radioligand concentrations until saturation occurred at 0.3 nmol/l. Similarly [^{3}H]AF-DX 384 was saturable at 0.2 nmol/l. In contrast, nonspecific binding increased proportionally to the applied radioligand. The saturation of specific binding of [^{3}H]PZ and [^{3}H]AF-DX 384 at low concentrations (nanomolar) to M_1 and M_2 cholinoceptors, respectively, is consistent with pharmacologically specific binding. Figure 2 depicts typical Scatchard analysis for the binding of [^{3}H]PZ and [^{3}H]AF-DX 384 to M_1 and M_2 cholinoceptors, respectively. Computer analysis of the data [11–13] indicated a single, high affinity binding site for both radioligands. The K_D for [^{3}H]PZ was found to be 0.361 ± 0.063 nmol/l with a B_{max} of 54.2 ± 17.8 fmol/mg protein (n = 12). For [^{3}H]AF-DX 384 the K_D was 0.228 ± 0.004 nmol/l with a B_{max} of 31.8 ± 4.4 fmol/mg protein (n = 6).

For displacement studies, 0.229 nmol/l [^{3}H]PZ and 0.078 nmol/l [^{3}H]AF-DX 384 were incubated with varying concentrations (0.5–30 mmol/l of THA) under the previous conditions. The results were analysed by Hill plot to obtain the Hill coefficient (n_H). The inhibition constant (K_i) was calculated from IC_{50} values using the Cheng and Prusoff equation [14] (table 1). A sample data set was used for the calculation of K_i with the Munson and Rodbard correction [15] applied. The value obtained was identical to that from the Cheng and Prusoff equation. This is due to the fact that the bound to free ratio of ligand was relatively low, which renders it unnecessary to

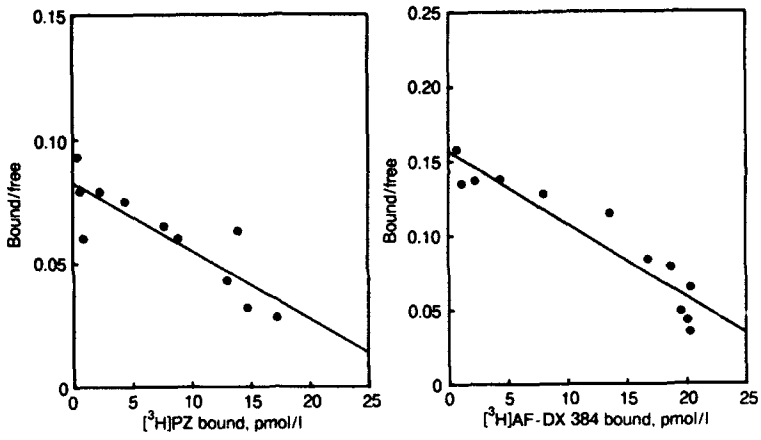

Fig. 2. Representative Scatchard analysis of specific [^{3}H]PZ and [^{3}H]AF-DX 384 binding to guinea pig membranes.

apply the Munson and Rodbard correction in our case [15].

Table 2 depicts the dissociation constants for [^{3}H]PZ and [^{3}H]AF-DX 384 in the presence and absence of THA (0–1,000 μmol/l). THA significantly slowed down the dissociation of [^{3}H]PZ from the M_1 sites. The same could not be said in the case of [^{3}H]AF-DX 384 as only minimal reduction in the dissociation constants was observed on increasing concentrations of THA.

Discussion

It has been suggested [13] that studying the effects of allosteric modulators on individual receptor subtypes is hindered when using tissues containing a heterogeneous mixture of M cholinoceptors. We have found that our results do not reflect a discrepancy between membranes prepared from either whole brain or regions. The K_D for whole brain homogenates for [^{3}H]PZ was 0.361 ± 0.063 nmol/l compared with that obtained using cerebral cortex, 0.381 ± 0.025 nmol/l. The differences are insignificant ($p > 0.05$). For the purpose of maximising receptor concentration for binding studies, we decided to use whole brain membranes for both M_1 and M_2 subtype assays.

While the K_D for [^{3}H]PZ reported in our studies (0.361 ± 0.063 nmol/l) is lower than others quoted in the literature (e.g. 35 nmol/l using rabbit hippocampi [5] and 3 nmol/l using rat cerebral cortex [4]), the difference may not be solely explained by interspecies variation. Pedder et al. [12] have concluded that different buffer systems and assay temperatures can markedly alter the affinity of antagonists such as gallamine and AF-DX 116 for their receptor subtypes. However, the data for

Table 1. Displacement of [^{3}H]PZ by THA and [^{3}H]AF-DX 384 by THA and MTA

Radioligand	Competitor	K_i, µmol/l	n_H	n
[^{3}H]PZ	THA	1.52 ± 0.12	1.58 ± 0.06**	5
[^{3}H]AF-DX 384	THA	2.42 ± 0.12	1.51 ± 0.02**	7
[^{3}H]AF-DX 384	MTA	0.024 ± 0.006	0.89 ± 0.01**	6

Values of K_i and n_H are means ± SEM. Student's t test was used to test whether n_H was significantly different from unity; ** $p < 0.01$. K_i = Inhibition constant; n_H = Hill coefficient; n = number of experiments.

pirenzepine indicate that its affinity for the M_1 cholinoceptor subtype is not adversely affected by either temperature or ionic strength of the buffer. In contrast, the affinity of pirenzepine for the M_1 cholinoceptor subtype increases when the receptors are solubilised in detergents such as digitonin and CHAPSO. Whether the low K_D values we reported here are a result of our specific assay conditions or some other factors remains to be determined.

The Hill coefficients (n_H) (1.58 for [^{3}H]PZ and 1.51 for [^{3}H]AF-DX 384) were significantly larger than unity. This implies that positive cooperativity may be part of the binding process and reflects the non-competitive nature of the inhibition of THA at both the M_1 and M_2 cholinoceptors. Allosteric interactions by THA at these M cholinoceptor subtypes have been suggested [2]. The K_i values of THA at both receptors were similar. THA is therefore not an effective antagonist to discriminate between M cholinoceptor subtypes.

Dissociation studies investigating the effect of THA on radioligand binding were performed. The dissociation was assumed to follow first order kinetics. The dissociation constants for each radioligand at varying THA concentrations are shown in table 2. Increasing concentrations of THA progressively blocked the dissociation of [^{3}H]PZ from the M_1 cholinoceptor supporting the contention that THA binds to an allosteric site [9].

Table 2. Dissociation constants for [^{3}H]PZ and [^{3}H]AF-DX 384 at varying THA concentrations

Tacrine µmol/l	k, min^{-1}	
	[^{3}H]PZ	[^{3}H]AF-DX 384
0	0.019 ± 0.0004 (6)	0.006 ± 0.001 (3)
1	–	0.007 ± 0.001 (3)
50	0.016 ± 0.0003** (3)	–
100	0.013 ± 0.003* (4)	0.004 ± 0.003 (3)
500	0.003 ± 0.001** (3)	–
1,000	–	0.004 ± 0.001 (3)

k (min^{-1}) is the dissociation constant determined as the slope of the plot of ln (B/Bo) vs. t, where B_0 is specific binding at time zero and B is specific binding at time t; k is presented as the mean ± SEM. The numbers in parentheses are the number of experiments performed. Student's t test was used to test if k for THA treatment was significantly different to k of control; * $p < 0.05$; ** $p < 0.01$.

Pearce and Potter [5] have reported that dissociation of the M_2 agonist [^{3}H]oxotremorine-M was not affected by tacrine. Similarly, we have found that THA has no effect on [^{3}H]AF-DX 384 dissociation. A comparison of the dissociation constants (table 2) indi-

cates that there is no significant difference between the data obtained for the control and varying THA concentrations.

Our data indicate that while THA affinity for M_1 and M_2 cholinoceptors is similar, the nature of its allosteric interaction is much different. It has been reported that muscarinic receptor subtypes may be associated with different allosteric binding sites [7, 13]. The binding of a modulator at these allosteric sites coupled to the primary sites can cause specific conformational changes which may facilitate or slow the binding of ligands to these receptor sites [5, 16]. We therefore propose that THA interaction at an allosteric site is determined by the specificity of the site and is not a function of THA itself.

We then compared the effects of MTA at M_2 cholinoceptors with those of THA. MTA is a selective M_2 cholinoceptor antagonist which discriminates between M_2 cardiac and M_1 cortical cholinoceptors in binding assays [9]. Although most studies on muscarinic actions of MTA have been carried out with cardiac and cerebellar tissues [17], we used whole brain membranes for consistency between assays.

[^{3}H]AF-DX 384 displacement studies were performed as previously described. An MTA concentration range of 0.01–0.4 mmol/l was used. The K_i was calculated to be 0.024 ± 0.006 mmol/l with an n_H of 0.89 ± 0.01 (n = 6). The n_H value of 0.89 is significantly different from unity suggesting negative cooperativity. In addition, MTA was 100 times more potent an inhibitor than THA at M_2 cholinoceptors. Giraldo et al. [17] have suggested that MTA exhibits both competitive and allosteric behaviour at M_2 cholinoceptors. Our results suggest that MTA interaction at M_2 subtypes is not consistent with a simple competitive model. The higher binding potency for MTA at M_2 sites may be related to its linear structure [9] which could have better access to the receptor than the ring structure of THA.

In conclusion, we found that THA has similar affinity for M_1 and M_2 cholinoceptor subtypes in guinea pig brain membranes. THA is also a less potent antagonist than MTA at the cardiac M_2 subtypes. It is also an allosteric modulator at a site associated with the M_1 subtype, as evidenced by its ability to slow the [^{3}H]PZ dissociation. No such allosteric effect was observed for M_2 cholinoceptors.

References

1 Summers WK, Majovski LV, Marsh GM, et al: Oral tetrahydroaminoacridine in long-term treatment of senile dementia, Alzheimer type. N Engl J Med 1986;315:1241–1245.

2 Freeman SE, Dawson RM: Tacrine: A pharmacological review. Prog Neurobiol 1991;36:257–277.

3 Freeman SE, Lau W-M, Szilagyi M: Blockade of a cardiac K^+ channel by tacrine: Interactions with muscarinic and adenosine receptors. Eur J Pharmacol 1988;154:59–66.

4 Flynn DD, Mash DC: Multiple in vitro interactions with and differential in vivo regulation of muscarinic receptor subtypes by tetrahydroaminoacridine. J Pharmacol Exp Ther 1989;250:573–581.

5 Pearce BD, Potter LT: Effects of tetrahydroaminoacridine on M_1 and M_2 muscarinic receptors. Neurosci Lett 1989;88:281–285.

6 Alonso R, Kan JP, Worms P, et al: Effects of repeated administration of tetrahydroaminoacridine (THA) on muscarinic receptor subtypes in the rat brain. Neurochem Int 1990; 17:457–465.

7 Potter LT, Ferrendelli CA, Hanchett HE, et al: Tetrahydroaminoacridine and other allosteric antagonists of hippocampal M_1 muscarinic receptors. Mol Pharmacol 1989;35:652–660.

8 Entzeroth M, Mayer N: Labelling of rat heart muscarinic receptors using the new M_2 selective antagonist [^{3}H]AF-DX 384. Biochem Pharmacol 1990;40:1674–1676.

9 Melchiorre C, Quaglia W, Picchio MT, et al: Structure-activity relationships among methoctramine-related polymethylene tetraamines. Chain-lengths and substituent effects on M-2 muscarinic receptor blocking activity. J Med Chem 1989;32:79–84.

10 Hartree EF: Determination of protein: A modification of the Lowry method that gives a linear photometric response. Anal Biochem 1972;48:422–427.

11 Burgeisser E: MxN-FIT, and IBM-PC Programme for the Analysis of Complex Binding Data according to the Law of Mass Action. Rheinfelden, Bureco, 1988, pp 1–111.

12 Pedder EK, Phike E, Poyner D, et al: Modulation of the structure-binding relationships of antagonists for muscarinic acetylcholine receptor subtypes. Br J Pharmacol 1991;103: 1561–1567.

13 Lee NH, El-Fakahany EE: Allosteric interactions at the m_1, m_2 and m_3 muscarinic receptor subtypes. J Pharmacol Exp Ther 1991;256:468–479.

14 Cheng YC, Prusoff WH: Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition of an enzymatic reaction. Biochem Pharmacol 1973;22:3099–3108.

15 Munson PJ, Rodbard D: An exact correction to the 'Cheng-Prusoff' correction. J Recept Res 1988;8: 533–546.

16 Hunter AJ, Murray TK, Jones JA, et al: The cholinergic pharmacology of tetrahydroaminoacridine in vivo and in vitro. Br J Pharmacol 1989; 98:79–86.

17 Giraldo E, Micheletti E, Montagna E, et al: Binding and functional characterisation of the cardioselective muscarinic antagonist methoctramine. J Pharmacol Exp Ther 1988;244:1016–1020.

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